Reference: *Biol. Bull.* **202:** 74–85. (February 2002)

Specificity of a Model Cnidarian-Dinoflagellate Symbiosis

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Abstract. To understand the flexibility of symbiotic associations in coral reefs, we investigated the specificity of the *Aiptasia* (cf. *insignis*)-*Symbiodinium* association in the laboratory by rendering the anemones aposymbiotic and inoculating them with different isolates of *Symbiodinium*. Infective algal symbionts were monitored over 3 months by re-isolation and identification using denaturing-gradient gel electrophoresis and sequence comparison of their amplified 18S rRNA hypervariable V1 + V2 gene region. Despite similarity in their external morphology, the algal isolates differed in their infectivity towards the host. Within days of single-isolate inoculation, aposymbiotic anemones formed associations with fresh or cultured isolates (clade B) from the anemones *Aiptasia* sp. or *A. tagetes*, respectively. They associated to a limited extent with cultured isolates (clade A) from the tridacnids *Tridacna crocea* or *Hippopus hippopus*, and not at all with a cultured isolate (clade C) from the stony coral *Montipora verrucosa*, nor with a free-living isolate (clade A) from subtidal sands. Aposymbiotic anemones inoculated with a mixture of all isolates had only the anemone taxon as their detectable symbionts. Re-inoculation of induced symbioses with a mixture of all isolates and incubation with wild anemones showed that the initial induced symbioses with the anemone taxon were stable. Anemones originally infected with tridacnid isolates either additionally acquired the anemone taxon or had the former outgrown by the latter. These results demonstrate the presence of a host-symbiont recognition mechanism, and possibly competition among potential algal symbionts in the *Aiptasia-Symbiodinium* association. Here we present a method that may be useful in monitoring the algal population dynamics in symbiotic corals in the field, along with an efficient method of rendering *Aiptasia* aposymbiotic for further laboratory investigation of *Aiptasia-Symbiodinium* symbioses.

Received 3 August 2000; accepted 16 November 2001.

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Introduction

Bleaching of symbiotic invertebrates in coral reefs, such as the reef-building corals, anemones, and giant clams, has increasingly been noted over recent years. This phenomenon, which results from the loss of the dinoflagellate symbionts (= zooxanthellae) or their pigment content, has mostly been associated with increasing water temperature (Brown and Ogden, 1993; Glynn, 1996), solar radiation (Gleason and Wellington, 1993; Shick *et al.*, 1996), or both (Drollet *et al.*, 1995). In some cases, bleaching leads to the death of the animal host; in other cases, the symbiosis and the host recover (Knowlton, 2001).

It is not clear how bleached invertebrate hosts recover. Perhaps at the end of the stressful period, any residual symbionts from bleached or unbleached (Hayes and Bush, 1990) host tissues are able to propagate to repopulate their hosts. It is also likely that bleached hosts acquire algal symbionts from those available in the water column (see Belda-Baillie *et al.*, 1999). The actual mechanism may be a combination of these (see also Jones and Yellowlees, 1997).

Recent use of molecular genetic markers has revealed the genetic diversity present among members of the predominant symbiotic genus *Symbiodinium*, which would otherwise be difficult to distinguish morphologically from one another. Analysis of patterns of restriction fragment length

polymorphism (RFLP) and sequences of their partial 18S rRNA gene revealed that the symbiotic dinoflagellates of many cnidarians form three distinct clades: A, B, and C. Similar algal symbionts associate with dissimilar hosts (Rowan and Powers, 1991a, b). Furthermore, the dominant Caribbean stony corals *Montastraea annularis* and *M. faveolata* have been found to simultaneously host *Symbiodinium* taxa from clades A, B, and C. These taxa are distributed within the host colonies according to gradients of environmental irradiance (Rowan *et al.*, 1997). Recently, distinct functional differences have also been shown among the clades' ability to synthesize mycosporine-like amino acids under the influence of ultraviolet and photosynthetically active radiation (Banaszak *et al.*, 2000).

An increasing appreciation of the genetic and functional diversity of *Symbiodinium* has stimulated interest in the ability of bleached hosts to accept different taxa of *Symbiodinium* and in whether they form stable symbioses. Attempts to address these and related questions in the anemone and clam symbioses, for example, have been hampered by the limited availability of tools that would easily distinguish and monitor any competition between diverse algal populations inside the animal host (*e.g.*, Kinzie and Chee, 1979; Schoenberg and Trench, 1980; Fitt, 1985; Davy *et al.*, 1997; Schwarz *et al.*, 1999; Belda-Baillie *et al.*, 1999).

In this study, we reexamined symbiosis specificity by comparing the relative infectivity of different isolates of *Symbiodinium* with artificially bleached *Aiptasia* sea anemones. These cnidarian hosts have been widely used as test animals for studies of cnidarian-dinoflagellate interactions. Here we used denaturing-gradient gel electrophoresis (DGGE, Fischer and Lerman, 1979) of the hypervariable V1 + V2 region of the alga's 18S rDNA to distinguish between *Symbiodinium* taxa before and after infection of aposymbiotic anemones. DGGE is a fast and inexpensive means of physically separating sequence variants. Used in combination with PCR primers designed to conserved regions of the 18S rRNA gene from *Symbiodinium* (see Carlos *et al.*, 2000), this method allows the study of communities of algal symbionts within marine invertebrates such as *Aiptasia*. Sequencing of the PCR products separated by DGGE permits identification of members of the symbiont community and analysis of phylogenetic relationships between different taxa. By attaching a high-temperature melting domain of GC-rich sequence to the DNA fragment, the technique can detect nearly 100% of all possible sequence variation (Sheffield *et al.*, 1989). This approach can therefore detect a mixture of taxonomically distinct or closely related *Symbiodinium* within the animal host (see Carlos *et al.*, 2000).

DGGE has been successfully used in profiling complex bacterial communities (Muyzer *et al.*, 1993; Øvreås, 2000) and eukaryotic organisms in aquatic environments (Van Hannen *et al.*, 1998), as well as in estimating the relative densities of uncultured bacterial populations (Watanabe and Harayama, 1998). Carlos *et al.* (2000) recently used a variant of this technique, temperature-gradient gel electrophoresis (Rosenbaum and Riesner, 1987), in assessing the genetic diversity of *Symbiodinium* in individual clam and anemone hosts. The same approach can be used to monitor the field dynamics of algal populations in coral reefs (our unpublished data). In this study, we successfully used DGGE analysis for the first time in the investigation of cnidarian-dinoflagellate interactions, particularly the specificity of the association.

Materials and Methods

Induced bleaching of Aiptasia *anemones*

"Wild" symbiotic *Aiptasia* (cf. *insignis*) (*ca.* 1-cm oral-disc size), descendants of a single specimen originally from a coral reef in Okinawa, were carefully collected from an indoor aquarium containing flowing, sand-filtered seawater from a nearby bay (see GenBank accession AY046885 for our 18S rDNA sequence of this study organism). The aquarium was heated to about 25°C, and lit on a 12 h: 12 h photoperiod with approximately 80 μmol photons $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR) from daylight FL 20S-E fluorescent tubes. Except in the case of the infection trials described below, anemones were not fed. In the indoor aquarium the anemones regularly proliferated under fluorescent lights and in the presence of the sand-filtered seawater.

Individual anemones were placed in 30-ml glass vessels with translucent lids. Each glass vessel contained 25 ml of autoclaved 0.45-μm filtered seawater (FSW). The following day, after all anemones had attached themselves inside the glass vessels, the anemones were placed in an incubator at a temperature of 25.0 $\pm$ 0.1°C and PAR of 78–82 μmol photons $m^{-2}s^{-1}$ on a 12 h: 12 h photoperiod. After an acclimation period of 2 weeks, replicate anemones were randomly distributed to one of four treatments with controlled temperature and light as follows: (1) 25°C with 80 μmol photons $m^{-2}s^{-1}$ PAR (light control), (2) 25°C in the dark (dark control), (3) 32°C in the dark (moderate heat stress), and (4) 35°C in the dark (high heat stress). All treatments were applied inside a multichamber incubator (Bio multi incubator model LH-30-8CT). The treatments chosen above were based on preliminary experiments which showed that temperatures below 35°C in the presence of light were ineffective in rendering the anemones aposymbiotic, whereas temperatures of 35°C or above in the presence of light were lethal to the anemones. Throughout the bleaching experiment, the FSW medium was replaced twice a week.

At the beginning of the bleaching experiment and at subsequent regular intervals, the chlorophyll *a* (Chl *a*) content of the anemones was monitored to estimate the decline

in density of their algal symbionts. Replicate anemones ($n = 4$) from each treatment were separately rinsed in distilled water and homogenized at 5000 rpm for 60 s by beadbeating with a sterile 6-mm stainless steel ball bearing in a 2-ml tube containing FSW (Biospec mini-beadbeater model 3110BX). The algal fraction was separated from the animal fraction by centrifugation at 1000 × *g* for 5 min. To remove any remaining host tissues, the algal pellet was resuspended in FSW before being recovered by a brief centrifugation (10,000 × *g* for ~1 s). This wash procedure was repeated once more before the algal pellet was again recovered by centrifugation (10,000 × *g* for 1 s) and stored frozen at −20°C. The beadbeating procedure did not result in the release of algal pigments into the homogenate; hence, it was assumed that algal cells remained intact throughout the procedure.

Chl *a* was later extracted from the algal cells by beadbeating for 30 s with about 250 μl of 800-μm glass beads in 90% acetone and incubating at 4°C for 24 h. The sample was then centrifuged (2000 × *g* for 10 min), and the absorbance of the supernatant was read at 750, 664, and 630 nm. Chl *a* concentration was quantified using the equations of Jeffrey and Humphrey (1975). When Chl *a* could no longer be detected spectrophotometrically in the anemones at the final sampling, the anemones were examined under a fluorescence microscope (Olympus SZX12) to verify the absence of red fluorescence emanating from any residual algal symbionts. Finally, the total DNA from whole *Aiptasia* individuals (including any remaining symbiotic algae) was extracted and polymerase chain reaction (PCR) carried out using zooxanthellae-specific primers (described below) to determine if they were completely aposymbiotic.

Induced symbiosis between aposymbiotic anemones and Symbiodinium *isolates*

After the development of an efficient means of rendering the anemones aposymbiotic, more anemones in individual vessels were induced to bleach within 1 month. They were acclimated to normal conditions (25°C, 80 μmol photons $m^{-2}s^{-1}$ PAR) for 2 weeks, and then a few representatives were checked for the complete absence of algal symbionts by examination under a fluorescence microscope.

Meanwhile, log-phase cultures of the following isolates of *Symbiodinium* were harvested and washed in FSW by centrifugation at 1000 × *g* for 5 min: (1) TC2A, a clonal isolate from the tridacnid clam *Tridacna crocea* (see isolate PHQU TC2A from Baillie *et al.*, 1998); (2) HH2A, a clonal isolate from the tridacnid clam *Hippopus hippopus* (see isolate PHSC HH2A from Baillie *et al.*, 1998); (3) HA3-5, a clonal isolate of a free-living isolate from Hawaiian subtidal sands (see Carlos *et al.*, 1999); (4) CS164, a clonal isolate from the anemone *Aiptasia tagetes* (culture purchased from the Commonwealth Scientific and Industrial Research Organisation [CSIRO], Australia); and (5) CS156, a clonal isolate from the stony coral *Montipora verrucosa* (culture purchased from CSIRO). In addition to these five clonal cultures, zooxanthellae from a specimen of *Aiptasia* sp. held in the aquarium were freshly isolated (*Aiptasia* FIZ) using the protocol described above. Analysis of the partial sequences of their 18S rDNA indicates that the isolates TC2A, HH2A, and HA3-5 belong to clade A of the *Symbiodinium* phylogeny (GenBank accession numbers: AF289268, AF289269, AB016572, respectively); CS164 (AF289270) and *Aiptasia* FIZ (AF289267) belong to clade B; and CS156 (AB016594) belongs to clade C (see also Carlos *et al.*, 1999, 2000). TC2A and HH2A further differ from one another by one nucleotide, judging from their internal transcribed spacer regions (ITS-5.8S-ITS2) (our unpublished data).

Except for a control group ($n = 10$ anemones), replicate aposymbiotic anemones were inoculated with one of the six isolates ($n = 10$ anemones for each isolate), while another group ($n = 10$ anemones) was inoculated with a mixture of all six isolates. Algal suspensions of the isolate being studied were squirted over the oral discs of the anemones at a final concentration of about 10^4 cells ml^{-1} anemone medium. To check for algal infection, the inoculated anemones were observed daily for a week at high magnification under a dissecting microscope (OM System). At 2, 4, 8, and 12 weeks after the initial inoculation, any infective symbionts were re-isolated from anemones ($n = 2$) from each treatment and frozen for identification and comparison with the inoculating isolates.

To determine whether the initially induced symbioses were stable, the remaining anemones ($n = 2$) from each treatment were re-inoculated once with a mixture of all the cultured isolates; in addition, each was incubated with a wild symbiotic anemone taken from aquarium stocks. This treatment ensured that the remaining anemones from each treatment had access to a wide range of possible algal symbionts, including the *Symbiodinium* that is regularly released by the wild anemones. After 1 month, the symbionts from anemones ($n = 2$ for each treatment) were re-isolated for identification. The experimental anemones were easily distinguishable from the wild symbiotic anemones by their relatively light color and small size.

Two sets of the infection experiment described above were carried out simultaneously. In the first set, the anemones were fed once a week with freshly hatched *Artemia nauplii*. In the second set, the anemones were unfed. This was to determine the effect of host nutritional status on the infectivity of the algal isolates and the stability of the induced symbioses. In both sets, the FSW medium was changed twice a week, a few hours after the anemones in the first set were fed.

Bleached anemones that appeared to remain aposymbiotic after exposure to algal isolates were examined for the

presence of algal cells by means of Chl *a* measurements, fluorescence microscopy, and PCR using zooxanthellae-specific primers (described below) to determine if they were completely aposymbiotic. Anemones were considered to have remained completely bleached only if they lacked Chl *a*, they had no fluorescent algal cells in their tissues, and their total DNA failed to produce a zooxanthellae-specific PCR product.

Extraction of DNA from anemones and from symbiotic and cultured algae

Total DNA from visibly bleached *Aiptasia* individuals (including any symbiotic algae present) was extracted by beadbeating each anemone (5000 rpm for 60 s with a sterile 6-mm stainless steel ball bearing) in a 2-ml tube containing 1.0 ml of proteinase K buffer (10 m*M* Tris; 100 m*M* EDTA, 1.0% SDS). The homogenate was then incubated at 40°C overnight with proteinase K (0.1 mg ml^{-1}), the protein fraction extracted twice with an equivalent volume of buffered phenol, and the phenol removed by extraction twice with an equivalent volume of chloroform. DNA was precipitated by addition of 2.5 volumes of absolute ethanol and 0.1 volume of 3 *M* sodium acetate (pH 6.1), and incubation at −20°C for at least 30 min. Precipitated DNA was recovered by centrifugation at 10,000 × *g* for 15 min, washed with 70% ethanol, and dried in a vacuum centrifuge for 2 min. Dried DNA was dissolved in 100 μl TE buffer (10 m*M* Tris; 1 m*M* EDTA, pH 8.0) and quantified by measuring its absorbance at 260 nm on a DU 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

For DNA extraction from cultured and symbiotic algae, algal pellets were prepared as described earlier. Each algal pellet was suspended in 1.0 ml of proteinase K buffer, transferred to a 2-ml tube containing about 750 μl of 800-μm glass beads, and lysed at 5000 rpm for 60 s in a mini-beadbeater. DNA was then extracted from the lysate as described above, using the proteinase K method.

Molecular genetic identification of infective Symbiodinium *taxa*

PCR was used with the following zooxanthellae-specific primers (Carlos *et al.*, 2000) to amplify the hypervariable V1 + V2 region of the alga's 18S rRNA gene: z8 (forward) 5′-GTCTCAAAGATTAAGCCATG-3′; zPV2R (reverse) 5′-GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCTCCGTTACCCGTCATTGCC-3′ (annealing region underlined). A 40-bp GC-rich sequence or GC-clamp (Sheffield *et al.*, 1989) was attached to the 5′ end of the reverse primer.

AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA) was used for all PCR amplifications. PCRs (20 μl) contained 10 m*M* Tris-HCl, 50 m*M* KCl, 0.001% (w/v) gelatin, 1.5 m*M* $MgCl_2$, 150 μ*M* of each dNTP, 0.5 U of AmpliTaq Gold, 8 pmol of forward primer, 8 pmol of reverse primer, and between 1 and 10 ng of DNA. Amplification reactions were run in a TP240 thermal cycler (Takara Biomedicals, Japan) using the following thermal program: 94°C for 12 min; 40 cycles of 94°C for 30 s, 50°C for 30 s, 68°C for 2 min; and 72 C for 15 min. These reaction conditions were used to amplify any zooxanthellae 18S rDNA present in the total DNA from bleached anemones and also from the DNA extracted from algal pellets. The presence of the PCR product (*ca.* 370 bp) was confirmed by electrophoresis on a 1.5% agarose gel.

To resolve different amplified 18S rDNAs derived from different *Symbiodinium* taxa within the anemone host, 5 μl of the PCR products was loaded on a parallel DGGE gel using the Bio-Rad Dcode universal mutation detection system. The PCR products were resolved on a 6% acrylamide gel (acrylamide/bis 37.5:1) using a 0%–100% denaturing gradient (0–7 *M* urea and 40% v/v formamide) at 58°C, and run at 150 V for 5 h. The gel was later stained in SYBR Green II (Molecular Probes) for 20 min and viewed on a UV transilluminator. Each vertical lane on the gel represents a "population profile," or a pattern of bands with each band representing a different taxon of *Symbiodinium*. Distinct bands containing the DNA fragment were excised, immersed in 100 μl TE buffer, and shaken at 30°C overnight. Eluted DNA was precipitated in absolute ethanol with 0.3 *M* sodium acetate, washed in 70% ethanol, dried in a vacuum centrifuge, and redissolved in 20 μl TE buffer. The purified DNA fragment was then re-amplified using the same set of primers and PCR conditions. The nucleotide sequences of the amplified products were determined using the ABI PRISM dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems) and the nested sequencing primers z9 (forward): 5′-ATGCATGTCTCAGTATAAGC-3′; zSV2R (reverse): 5′-CTCCGTTACCCGTCATTGCC-3′ (Carlos *et al.*, 2000). The sequencing reaction products were read on a 377 DNA sequencer (PE Applied Biosystems).

To identify the algal symbionts from the infected anemone hosts, the nucleotide sequences of their DGGE bands and those of *Symbiodinium* clade representatives from the Genbank database were aligned using the multiple sequence alignment program Clustal W version 1.7 (Thompson *et al.*, 1994). The 18S rDNA V1 + V2 sequences, excluding ambiguous regions (gaps, insertions, flanking primer regions), consisted of 292 aligned sites. To confirm the clade affiliation of the algal symbionts, phylogenetic reconstruction based on the neighbor joining distance method (Saitou and Nei, 1987) was carried out using Clustal W version 1.7 with a Kimura two-parameter distance matrix. Clade reliability was estimated by 1000 times bootstrap resampling of the aligned data set. This method was used to establish clade affiliations, since phylogenetic reconstruction based on 18S rDNA sequence has been shown by Carlos *et al.* (1999) to

result in the reconstruction of *Symbiodinium* phylogenies that are consistent with the clade A, B, and C groups derived from RFLP data. In addition to the 18S rDNA sequence, phylogenetic reconstruction based on the internal transcribed spacer region (Baillie *et al.*, 2000b) and partial RUBISCO sequence (our unpublished data) also recovers phylogenies consistent with clades derived from RFLP data.

Electron microscopy observations of the external morphology of Symbiodinium *isolates*

Log-phase algal cultures of the different strains of *Symbiodinium* were harvested, washed, and resuspended in FSW as described above. One volume of each algal suspension was fixed for 2 h at room temperature with three volumes of 3.3% glutaraldehyde in 0.067 *M* Na-cacodylate buffer (pH 7.2) containing 1.33% NaCl. The final concentrations of glutaraldehyde, NaCl, and cacodylate were 2.5%, 1.0%, and 0.05 *M*, respectively. The fixed cells were serially dehydrated in ethanol (30%, 50%, 75%, 90%, 95%, 100%, and 100%), and transferred into an isoamylalcohol:ethanol mixture (1:1) and then into 100% isoamylalcohol. The cells were dried in a critical-point dryer, sputter-coated with Pt and Pd, and mounted on a specimen stub with double-sided tape. The cells were then observed under a Hitachi S-2500 scanning electron microscope.

Results

Induced bleaching of Aiptasia *anemones*

Figure 1 shows the Chl *a* content of the anemones over time, representing the temporal change in algal density in the different treatments. By day 10, all anemones had lost some Chl *a* or algal symbionts, with those at 35°C in darkness having the least amount of Chl *a* and looking the palest of all. By day 20, all anemones had lost more Chl *a* or algal symbionts and looked much paler, while those at 35°C in darkness had no detectable Chl *a* and appeared completely white. When examined under a fluorescence microscope, these bleached anemones (incubated at 35°C in the dark) had no detectable algal symbionts. When total DNA from bleached anemones was used in PCR using zooxanthellae-specific primers, no algal 18S rDNA could be amplified. These data demonstrate that anemones treated at 35°C in the absence of light for 20 days were free from algal symbionts. The bleaching treatment apparently did not harm the anemones, as indicated by their normal feeding response and continued asexual reproduction through pedal laceration.

Infectivity of different Symbiodinium *isolates*

A few minutes after inoculation, the algae could be seen in the anemone's translucent gastrodermal cavity. Although some motile *Symbiodinium* were present in the inocula, we did not observe motile forms actively swimming into the anemones. Anemones, however, showed feeding behavior immediately upon inoculation, using their tentacles to transfer algae into their oral discs. The algae became immobilized upon touching the anemones' tentacles. It was likely that infection by successful isolates (*i.e.*, the presence of coccoid algal cells inside the host tissues) occurred within a day of inoculation, because by day 2 algal symbionts could be seen spread throughout the transparent tissues around the oral disc and tentacles of the anemones (Table 1).

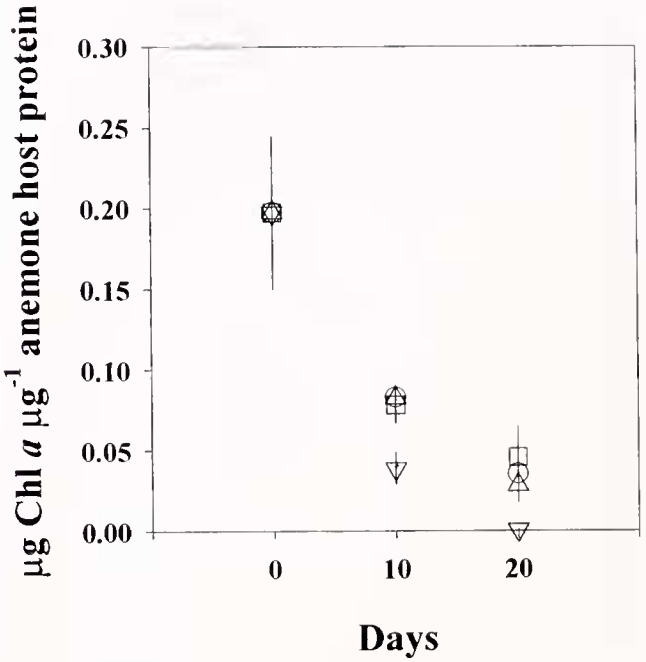

Figure 1. Mean chlorophyll *a* (Chl *a*) content (= algal density) (± SE, $n = 4$) of symbiotic *Aiptasia* anemones exposed to different light and temperature conditions to induce bleaching. (○ = 25°C in light; □ = 25°C in darkness; △ = 32°C in darkness; ▽ = 35°C in darkness.)

After 3 months, uninoculated control anemones remained aposymbiotic (as determined by microscopic observation, Chl *a* measurement, and PCR analysis). Those inoculated with a clade C isolate (CS156) or a clade A isolate (free-living HA3-5) also remained aposymbiotic. This was despite the continued presence for months of motile *Symbiodinium* in the water column and, mostly, on the bottom of the glass vessels (even after cleaning and regular replacement of seawater). Such motile *Symbiodinium* were probably residual algae that could not be removed during cleaning of the vials.

Other anemones became visibly brown within 2–3 weeks. As seen by the naked eye and under the fluorescence microscope, of the anemones inoculated with clade A isolates from tridacnids (isolates TC2A and HH2A), the HH2A anemones were infected with less-dense algal populations than the TC2A anemones. Because of limited time and experimental animals, no attempt was made to quantify the

Table 1

Infectivity over time of different Symbiodinium *isolate on aposymbiotic* Aiptasia *anemones*

		Weeks following inoculation				
Algal strain (clade)	Original source of isolate	1	2	4	8	12
Control (none)	—	−	−	−	−	−
CS156 (C)	*Montipora verrucosa* (coral)	−	−		−	−
HA3-5 (A)	Free-living in subtidal sand	−	−	−	−	−
Hh2a (A)	*Hippopus hippopus* (tridacnid)	$+^{day4}$	+	+	+	++
Tc2a (A)	*Tridacna crocea* (tridacnid)	$+^{day3}$	+	++	++	+++
CS164 (B)	*Aiptasia tagetes* (anemone)	$+^{day2}$	++	+++	+++	+++
Aiptasia FIZ (B)	*Aiptasia* sp. (anemone)	$+^{day2}$	++	+++	+++	+++
Mixture of above	All of above	$+^{day2}$	++	+++	+++	+++

[−] = no infection; [day#] = observed time of initial infection; [+] = extent of infection.

relative growth of the algal populations in different treatments. Anemones inoculated with the clade B isolate (CS164) or *Aiptasia* FIZ (clade B algae) from anemones were successfully infected, with dense algal populations in the hosts' tentacles and basal columns. Those inoculated with a mixture of all isolates were also successfully infected. Infectivity of the different algal isolates, as determined by microscopic observation, was the same whether the previously bleached hosts were fed or unfed.

Comparison of the external morphology of different Symbiodinium *isolates*

The coccoid forms of *Symbiodinium* isolates seen under a scanning electron microscope appeared similar, except for size (data not shown). The same was true for all motile forms. No motile forms were observed among symbionts that were freshly isolated from *Aiptasia*. It is difficult, if not impossible, to distinguish one isolate from the other based on their external morphology (*cf.*, Trench and Blank 1987, LaJeunesse, 2001).

Stability of induced symbioses over time and upon re-inoculation

Figure 2 shows the population profiles of the *Symbiodinium* isolates used for inoculating the anemones, the algal populations present in the anemones 1 to 3 months after initial inoculation, and the algal populations 1 month after re-inoculation with a mixture of algal isolates and incubation with wild symbiotic anemones. DGGE analysis of the clonal cultures and FIZ revealed a single band for each of these isolates, and further revealed PCR products that had different DNA sequences. The differences among the 18S rRNA gene copies in the clonal cultures and *Aiptasia* FIZ are therefore negligible, and DGGE analysis was able to resolve differences between the 18S rRNA gene sequences of the *Symbiodinium* clades used in this study. The identical 18S rDNA V1 + V2 sequences of the two clade A isolates from clams are reflected in the same mobility of their single bands. Although difficult to see from the figure (Fig. 2), mixtures of the two different clade A sequences (HA3-5 and

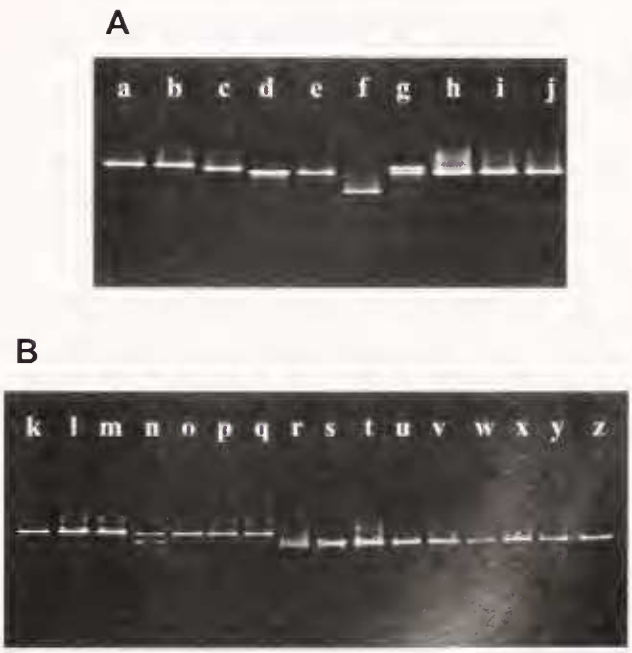

Figure 2. Denaturing-gradient gel electrophoresis (DGGE) profiles of inoculating *Symbiodinium* isolates and re-isolated algal symbionts based on their amplified 18S rDNA V1 + V2 region. Panels A and B represent separate DGGE gels. Panel A: lanes a–g are the respective DGGE profiles of inoculating *Symbiodinium* isolates, namely, Tc2a (clade A), Hh2a (clade A), HA3-5 (clade A), CS164 (clade B), *Aiptasia* FIZ (clade B), CS156 (clade C), and Mixture (clades A-B-C), lanes h–j are the respective DGGE profiles of the symbionts re-isolated from anemones 1 and 3 months after initial inoculation with a mixture of strains, and 1 month after re-inoculation with the mixture. Panel B: lanes k–n respectively represent the original TC2A inoculum, symbionts re-isolated from anemones 1 month after initial TC2A inoculation, symbionts re-isolated from anemones 3 months after initial TC2A inoculation, and symbionts re-isolated from anemones 1 month after Mixture-re-inoculation. This pattern is repeated across the gel for HH2A (o–r), CS164 (s–v), and *Aiptasia* FIZ inoculation (w–z).

the clam isolates) can be separated by DGGE. The identical DNA sequences of the two clade B isolates from anemones (CS164 and *Aiptasia* FIZ) are also reflected in their same mobility under DGGE. The mixed inoculum had a four-band profile, reflecting the presence of two sequences in clade A, one in clade B, and one in clade C.

After 1 to 3 months of initial mixed-isolate inoculation, and a month after re-inoculation with mixed strains (including expelled symbionts from wild anemones), only clade B symbionts could be detected in the anemone hosts. After 1 to 3 months of inoculation, the clade A clonal isolate TC2A had an identical DGGE profile and amplicon sequence to that of the re-isolated symbionts from TC2A-inoculated anemones. After re-inoculation with a mixture of isolates, however, DGGE profiles revealed a second taxon—a clade B anemone symbiont— in the anemones. The clade A clonal isolate HH2A was also identical to re-isolated symbionts from anemones after 1 to 3 months of inoculation with HH2A. But after re-inoculation with a mixture of isolates, this clam taxon was outgrown, or possibly displaced, by a clade B anemone symbiont. The re-isolated symbionts from anemones initially inoculated with cultured or FIZ isolates from *Aiptasia* spp. remained identical to the original inoculum over time and after re-inoculation with mixed isolates. The nutritional status of the hosts affected their final size, with the unfed ones being much smaller than the fed ones, but had no discernible effect on the stability of the induced symbioses (data not shown).

The clade affiliation of the inoculating isolates and infective *Symbiodinium* taxa was confirmed by phylogenetic reconstruction with known *Symbiodinium* clade representatives (data not shown; see Carlos *et al.*, 1999, 2000). In Figure 3, the 18S rDNA V1 + V2 sequences of the inoculating and infective taxa in the previously aposymbiotic anemones are compared. Anemones initially infected with the HH2A (clade A) isolate from a clam appeared to have eventually lost this taxon; they acquired an anemone taxon (clade B) following re-inoculation. Anemones initially infected with the TC2A (clade A) isolate from a clam maintained this taxon, but acquired a second taxon from anemones (clade B) after re-inoculation. Anemones initially infected with cultured (CS164) or fresh (FIZ) anemone isolates (clade B) retained these taxa over time and after re-inoculation. Those inoculated with a mixture of all isolates had only the anemone taxon (clade B) as their detectable symbionts.

Discussion

This study demonstrates the specific nature of the association between *Aiptasia* (cf. *insignis*) and *Symbiodinium*. Some algal isolates were able to infect aposymbiotic anemones while others were not, even in the absence of competing taxa. In particular, symbiosis seemed to be favored between the *Aiptasia* hosts and algal isolates from identical or related anemones. Schoenberg and Trench (1980) found much earlier that aposymbiotic *A. tagetes* were more successfully infected by homologous isolates of *Symbiodinium* (from same host species) than by heterologous isolates (from different host species), some of which were unable to infect the anemones at all, even after 6 months of inoculation. The aposymbiotic planulae of the temperate anemone *Anthopleura elegantissima* were able to form an association with fresh isolates from a conspecific adult, but not with cultured *S. californium*, a species reported to occur in *A. elegantissima* (Banaszak *et al.*, 1993; see Schwarz *et al.*, 1999). In this study, *Aiptasia* hosts also associated to a limited extent with algal isolates from tridacnid clams. Indeed, a number of different marine invertebrates that host *Symbiodinium* were also reported to form heterologous associations under controlled conditions (see Kinzie, 1974; Kinzie and Chee, 1979; Schoenberg and Trench, 1980; Colley and Trench, 1983; Davy *et al.*, 1997). The results presented here provide further support for the presence of some host-symbiont recognition mechanism between the anemone host and its potential dinoflagellate symbionts (Kinzie, 1974; Kinzie and Chee, 1979; Schoenberg and Trench, 1980; Trench *et al.*, 1981; Colley and Trench, 1983; Davy *et al.*, 1997).

An all-or-none process of recognition suggested by Schoenberg and Trench (1980) for zooxanthellate symbioses may account for the observed preference of association between the *Aiptasia* hosts and some algal taxa over at least the short term. Also, in the *Hydra-Chlorella* symbiosis (reviewed by Schoenberg and Trench, 1980), it has been suggested that recognition follows nonspecific phagocytosis of a zoochlorella by a host digestive cell. Retention or expulsion of the alga is determined by interactions between the host vacuole's inside-surface receptors and the algal surface antigens that vary between algal isolates. In the coral *Fungia scutaria-Symbiodinium* association, dinoflagellates that entered the gastric cavity of aposymbiotic coral planulae were also observed to be phagocytosed by the host's endodermal cells (Schwarz *et al.*, 1999).

Davy *et al.* (1997) found that uptake of zooxanthellae from *Anthopleura ballii* into *Cereus pedunculatus* was lower than that for other zooxanthellae. They proposed that discrimination based on the surface characteristics of the algae might explain this phenomena. Although some workers have used morphological characteristics to describe several zooxanthellae species (see Trench and Blank, 1987; LaJeunesse, 2001), our SEM observations of the isolates in the present study showed that they have similar external morphology. There appears to be no observable correlation between symbiont morphology (as seen with SEM) and infectivity (see also Schoenberg and Trench, 1980). As others have proposed, it is possible that the recognition

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                          48
Hh2a inoculum             CATGTCTCAGTATAAGCTTTTACACGGCGAAACTGCGAATGGCTCATTAAAGCAGTTATAATTTATTTGATGG
"Hh2a" re-isolate         ----------------AG-C-A-T--T-------------------------------G-----------
Tc2a inoculum             ----------------TT-T-C-C--C-------------------------------A-----------
"Tc2a" re-isolate #1      ----------------TT-T-C-C--C-------------------------------A-----------
"Tc2a" re-isolate #2      ----------------AG-C-A-T--T-------------------------------G-----------
CS164 inoculum            ----------------AG-C-A-T--T-------------------------------G-----------
"CS164" re-isolate        ----------------AG-C-A-T--T-------------------------------G-----------
FIZ inoculum              ----------------AG-C-A-T--T-------------------------------G-----------
"FIZ" re-isolate          ----------------AG-C-A-T--T-------------------------------G-----------
"Mixture" re-isolate      ----------------AG-C-A-T--T-------------------------------G-----------

Hh2a inoculum             TCACTGCTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCACCAAAACCCAACTTCGCAGAAGGGTT
"Hh2a" re-isolate         -TG-------------------------------------------T-C--G---G----T-----------
Tc2a inoculum             -CA-------------------------------------------C-A--A---A----C-----------
"Tc2a" re-isolate #1      -CA-------------------------------------------C-A--A---A----C-----------
"Tc2a" re-isolate #2      -TG-------------------------------------------T-C--G---G----T-----------
CS164 inoculum            -TG-------------------------------------------T-C--G---G----T-----------
"CS164" re-isolate        -TG-------------------------------------------T-C--G---G----T-----------
FIZ inoculum              -TG-------------------------------------------T-C--G---G----T-----------
"FIZ" re-isolate          -TG-------------------------------------------T-C--G---G----T-----------
"Mixture" re-isolate      -TG-------------------------------------------T-C--G---G----T-----------

Hh2a inoculum             GTATTTATTAGATACAGAACCAACGCAGGCTCCGCCTGGT  TGTGGTGATTCATGATAACTCGATGAATCGT
"Hh2a" re-isolate         --G----------C----------C---------------CA--------------------T--C-------
Tc2a inoculum             --A----------A----------G---------------  --------------------C--T-------
"Tc2a" re-isolate #1      --A----------A----------G---------------  --------------------C--T-------
"Tc2a" re-isolate #2      --G----------C----------C---------------CA--------------------T--C-------
CS164 inoculum            --G----------C----------C---------------CA--------------------T--C-------
"CS164" re-isolate        --G----------C----------C---------------CA--------------------T--C-------
FIZ inoculum              --G----------C----------C---------------CA--------------------T--C-------
"FIZ" re-isolate          --G----------C----------C---------------CA--------------------T--C-------
"Mixture" re-isolate      --G----------C----------C---------------CA--------------------T--C-------
                                                                                          339
Hh2a inoculum             GTGGCTTGGCCGACGATGCATCTTTCAAGTTTCTGACCTATCAGCTTCCGACGGTAGGGTATGGGCCTACCGT
"Hh2a" re-isolate         -C---C-T----------G--A-----------------------------------------T----------
Tc2a inoculum             -T---T-G----------A--T-----------------------------------------G----------
"Tc2a" re-isolate #1      -T---T-G----------A--T-----------------------------------------G----------
"Tc2a" re-isolate #2      -C---C-T----------G--A-----------------------------------------T----------
CS164 inoculum            -C---C-T----------G--A-----------------------------------------T----------
"CS164" re-isolate        -C---C-T----------G--A-----------------------------------------T----------
FIZ inoculum              -C---C-T----------G--A-----------------------------------------T----------
"FIZ" re-isolate          -C---C-T----------G--A-----------------------------------------T----------
"Mixture" re-isolate      -C---C-T----------G--A-----------------------------------------T----------
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Figure 3. Comparison of the 18S rDNA V1 + V2 sequences of inoculating and infective *Symbiodinium* strains following denaturing-gradient gel electrophoresis. "HH2A" re-isolate refers to re-isolated symbionts from HH12A-inoculated anemones after re-inoculation with a mixture of *Symbiodinium* strains and incubation with symbiotic wild anemones; "TC2A" re-isolate refers to re-isolated symbionts from TC2A-inoculated anemones after re-inoculation with a mixture of isolates; and so on. (FIZ = freshly-isolated zooxanthellae from *Aiptasia* sp., [−] = conserved nucleotide, [ ] = missing nucleotide, number = nucleotide position on the 18S rRNA gene.)

between the anemone host and its algal symbionts occurs at the molecular level (for a review see Trench, 1997).

Previous studies by Lin *et al.* (2000) on the *Aiptasia pulchella-Symbiodinium* symbiosis have demonstrated that glycoproteins on the algal cell wall are crucial factors in the establishment of the association (see also Markell and Trench, 1993). It is possible that such surface glycoproteins or related molecules mediate recognition between *Aiptasia* and its potential symbionts (see Lin *et al.*, 2000). Failure of the clade C and free-living *Symbiodinium* isolates to infect *Aiptasia* suggests that they lack the appropriate recognition factors. On the other hand, the clam and clade B isolates appear to possess cell wall components suitable for host-symbiont recognition. Our future studies will investigate the presence of distinguishable surface epitopes on the cell walls of the *Symbiodinium* isolates used in this study.

This study also suggests that after initial recognition, infective taxa proliferate at different rates, with the homologous isolates (CS164 and FIZ from anemones) establishing higher symbiont densities than the heterologous isolates (TC2a and HH2a from clams). A similar observation was made by Davy *et al.* (1997) during the initial 8 weeks following infection of *Cereus pedunculatus* with homologous and heterologous zooxanthellae (see also Schoenberg and Trench, 1980). This effect may have been due to lower growth rates, higher levels of expulsion, or higher levels of digestion of the clade A taxa. Whatever the reason, it is interesting that although the clade A taxa have the same 18S rDNA sequence, their densities within infected anemones were different after 12 weeks. Since these clam isolates are almost certainly conspecific, this supports the idea that there is significant intraspecific genetic (and hence phenotypic) variation in these clade A symbionts from giant clams (see Belda-Baillie *et al.*, 1999; Baillie *et al.*, 2000 a, b).

After inoculation with a mixture of all *Symbiodinium* isolates, all anemones were found to host the clade B symbionts (CS164, *Aiptasia* FIZ, or both). In all but one case (that of TC2A), only the clade B symbionts were detected. This observation demonstrates another difference between these two clade A taxa from giant clams. Given the success of the anemone (clade B) isolates in outgrowing or displacing the HH2A symbionts and in co-inhabiting with the previously established TC2A symbionts, we can rank the isolates according to their ability to form an association with *Aiptasia* (and according to symbiont density after 12 weeks): HA3-5 and CS156 < HH2A < TC2A << CS164, *Aiptasia* FIZ.

The outgrowing or possible displacement of HH2A symbionts by the clade B symbionts could be a result of competitive interactions. In that case the clade B symbionts may be better adapted to growth within the same niche (the anemone) and are able to outcompete the clade A taxon. Alternatively, the same result might be seen if the host is able to distinguish between the two taxa, and the clade A taxon is preferentially digested or expelled even after forming an association with HH2A for many weeks. If the latter case is true, then recognition may occur during, as well as after, initial uptake. It also implies that there may be a range of cell-surface epitopes that a single host could recognize as "self" (see the review by Trench, 1997). On balance, the simplest explanation for the outgrowing or displacement of HH2A symbionts is competition for resources within the anemone host by two "recognized" symbionts. Efficient competitors are expected to maintain high specific growth rates and eventually, according to the principle of competitive exclusion, exclude less efficient taxa (see also Fitt, 1985).

The coexistence of the TC2A and clade B symbionts in the same anemones might be another form of competition between two symbiont taxa. It is arguable whether such an association with both taxa might persist indefinitely. The fact that TC2A was still present may reflect its ability to grow faster than the HH2A isolate when inside the anemone. It is also possible that different taxa may coexist over long periods in the same host by exploiting different niches or resources within that host (see Baillie *et al.*, 1998; Belda-Baillie *et al.*, 1999; Carlos *et al.*, 2000). Anemones, however, may offer algal symbionts fewer microhabitat variations than other hosts, such as the giant clams (see Carlos *et al.*, 2000). If different taxa coexist in the same niche, then diversity may be maintained according to Hutchinson's (1961) concept of diversity under nonequilibrium conditions (for a discussion, see Carlos *et al.*, 2000).

Distinct functional differences between clades A, B, and C *Symbiodinium* have recently been demonstrated (Banaszak *et al.*, 2000). All the A clades tested synthesized mycosporine-like amino acids under the influence of UV and PAR, but the B and C clades did not. In the present study, the limited number of clade representatives available for comparison precludes interpretation of any apparent discrimination between different clades (*i.e.*, selection of clade B over others). However, Schoenberg and Trench (1980) found the growth of infective algal symbionts to be directly related to their isoenzyme similarity coefficient relative to the native algal symbiont in the test host *Aiptasia tagetes*, but there was no correlation between infectivity of algal symbionts and the phylogenetic relatedness of the original hosts to the infected anemones.

In the present study, the aposymbiotic anemones were able to establish symbiosis with algal isolates from tridacnids (*Tridacna*, *Hippopus*), but not with an algal isolate from a stony coral (*Montipora verrucosa*). This is consistent with previous studies in which isolates from tridacnids were shown to successfully infect aposymbiotic *Aiptasia* (Kinzie and Chee, 1979). These observations suggest that the normally extracellular symbionts of giant clams (Norton *et al.*, 1992) are capable of an intracellular mode of existence in cnidarian hosts (see also Carlos *et al.*, 2000). It was ex-

pected, however, that the TC2A isolate from *T. crocea* would eventually have been outcompeted by the FIZ or CS164 isolate from *Aiptasia*, given our observation that only a clade B taxon was present in anemones that have been maintained with *T. crocea* in aquaria. Rowan and Powers (1991a) also found only clade B taxa in *Aiptasia pulchella* and *A. pallida* maintained in the laboratory, and in *Anthopleura elegantissima* collected from the field.

Our study used *Aiptasia* individuals arising from a single individual (*i.e.*, individuals with the same genotype), and thus we cannot say whether different host individuals would have the same preferences for symbionts. Nevertheless, the fact that *Aiptasia-Symbiodinium* associations can harbor clade B algae such as *Symbiodinium pulchrorum* (host = *Aiptasia pulchella*), *S. bermudense* (host = *A. tagetes*), and *Symbiodinium* sp. CS164 (host = *A. tagetes*) supports the idea that some *Aiptasia* species have an affinity for the clade B taxa. Previous analysis of the 18S rDNA sequence from our *Aiptasia* FIZ indicates that it is *S. pulchrorum* (Carlos *et al.*, 2000). On balance, it seems likely that the genotype of *Aiptasia* used in our study may be typical of the other *Aiptasia* spp. studied so far (inasmuch as it seems to have an affinity for the clade B symbionts). However, further study will be needed to determine if other *Aiptasia* genotypes have different symbiont specificities, as has been seen in the gorgonian *Plexaura kuna* (for example, see Goulet and Coffroth, 1997).

It is likely that similar specificity occurs in the anemones' close relatives, the reef-building corals. Schwarz *et al.* (1999) found that the aposymbiotic planular larvae of the scleractinian coral *Fungia scutaria* can be experimentally infected by *Symbiodinium* from clades A, B, and C (from *Cassiopeia xamachana*, *Aiptasia pallida*, and *F. scutaria*, respectively). However, the stability of the induced symbioses and competition between algal symbionts were not investigated. The prevalence of polymorphic symbioses in any given coral reef community (Rowan and Powers, 1991a; Carlos *et al.*, 1999) and the regular expulsion of algal symbionts (Steele, 1975; Hoegh-Guldberg *et al.*, 1987; Stimson and Kinzie, 1991; McCloskey *et al.*, 1996; Maruyama and Heslinga, 1997) suggest a common pool of diverse algal symbionts available for infection (see also Baillie *et al.*, 2000b). However, the specificity of the *Aiptasia-Symbiodinium* association and others in coral reefs suggests that, under normal environment conditions, existing symbiotic associations may be the most favored and stable of all possible partnerships, including those in which symbiont acquisition occurs through maternal inheritance. Nevertheless, the flexible nature of these symbiotic associations may be an adaptational advantage in changing environmental conditions (Buddemeier and Fautin, 1993; Rowan and Knowlton, 1995; Davy *et al.*, 1997).

It is curious that the only free-living *Symbiodinium* reported to date (Carlos *et al.*, 1999), the HA3-5 clade A isolate from Hawaiian subtidal sand, was unable to infect aposymbiotic *Aiptasia* anemones. Phylogenetic studies have shown that this isolate is closely related to the algal symbionts of some foraminiferans (Carlos *et al.*, 1999). However, HA3-5 has not as yet been shown to form associations with any marine invertebrates, so this isolate may be exclusively free-living. If this is indeed the case, there may be other free-living taxa in coral reefs that are genetically, though perhaps not morphologically, distinct from free-living stages of symbiotic algae. Whether the free-living stage of symbiotic taxa of *Symbiodinium* serves only as a dispersal mechanism remains to be seen. Further investigation of free-living *Symbiodinium* will improve our understanding of dinoflagellate population dynamics on coral reefs.

The decline in the algal Chl *a* content in the control anemones (25°C, with or without light) can be attributed to starvation and possibly to decreased shading relative to conditions in the aquarium. In the aquarium, the wild anemones had access to fresh particulates from flow-through seawater, and they were stocked at high densities. It is noteworthy that in rendering the anemones aposymbiotic, dark incubation for one month at elevated temperature was a quick and efficient means of removing algal symbionts, without the use of any chemicals and without apparent harm to the animal host. Elevated temperature (Iglesias-Prieto *et al.*, 1992; Warner *et al.*, 1996) must have acted synergistically with the absence of light to impair the photosynthetic mechanism of the algal symbionts, leading to their quick escape or removal. We believe that this technique for *Aiptasia* is a significant improvement over previously published protocols that required dark incubation for 2 to 3 years to completely remove the anemones' symbionts (Kinzie and Chee, 1979), or use for months of the photosynthesis inhibitor DCMU (3-[3,4-dichlorophenyl]-1,1-dimethylurea), which has been previously shown to be not completely effective (Schoenberg and Trench, 1980). Treatment at 35°C in the dark may not be suitable for inducing bleaching of other marine invertebrates, however. For example, the temperatures used in our study may be lethal to temperate hosts. In addition, a study of the temperate sea anemone *Anthopleura elegantissima* demonstrated that high temperature (20°C) decreased zoochlorellae density and had little effect on zooxanthellae (Saunders and Muller-Parker, 1997). Nevertheless, we believe that the method we used in this study will facilitate symbiosis research with *Aiptasia* sp. The application of a similar technique to other model zooxanthellae-associations, such as giant clams, will require further study and optimization.

This study also underscores the utility of appropriate molecular genetic tools in understanding the dynamics of invertebrate-dinoflagellate associations on coral reefs. In previous studies on the specificity of anemone-dinoflagellate symbioses (Schoenberg and Trench, 1980; Buddemeier

and Fautin, 1993; Rowan and Knowlton 1995; Davy *et al.*, 1997; Schwarz *et al.*, 1999), limited research tools precluded any investigation of competition between symbionts within the host. Even the use of a combination of allozyme and random amplified polymorphic DNA analyses in monitoring the progress of giant clams inoculated with different isolates only highlighted the limitation of these techniques in detecting taxonomic differences between *Symbiodinium* taxa (Belda-Baillie *et al.*, 1999). The present study has shown that DGGE of the variable regions of the small-subunit ribosomal gene (or other suitable genetic markers; see also LaJeunesse and Trench, 2000) is an appropriate tool for such studies. Although only DNA fragments up to 500 bp can be separated (Muyzer *et al.*, 1996), the technique is sufficient to identify the DGGE band and should simplify investigations of many important aspects of host-symbiont interactions on coral reefs.

Acknowledgments

We thank Ms. Nakako Hayashi for her assistance with DNA sequencing, Mr. Hiroshi Urata for his assistance with the SEM analysis, Ms. Mika Atsumi for preparing the culture media, and Ms. Rie Aburaki for her help in maintaining the experiments. The cultures of *Symbiodinium* isolates TC2A and HH2A were generously donated by Prof. Edgardo D. Gomez of the University of the Philippines Marine Science Institute. This work was conducted as part of the Industrial Science and Technology Frontier Program, with financial support from the Japanese Ministry of International Trade and Industry.

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